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PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

NOTIFICATION OF THE RECORDING
OF A CHANGE(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

To:

MURGITROYD & COMPANY
Chartered Patent Agents
373 Scotland Street
Glasgow G5 8QA
ROYAUME-UNI

| | |
|--|---|
| Date of mailing (day/month/year) 15 October 2001 (15.10.01) | IMPORTANT NOTIFICATION |
| Applicant's or agent's file reference P2662PC/TIPD | |
| International application No. PCT/GB00/03223 | International filing date (day/month/year) 18 August 2000 (18.08.00) |

| | |
|---|--|
| 1. The following indications appeared on record concerning: | |
| <input type="checkbox"/> the applicant | <input type="checkbox"/> the inventor <input checked="" type="checkbox"/> the agent <input type="checkbox"/> the common representative |
| Name and Address DUMMETT, Thomas, Ian, Peter Dummett Copp 25 The Square Martlesham Heath Ipswich IP5 3SL United Kingdom | State of Nationality |
| | State of Residence |
| | Telephone No. 01473 660600 |
| | Facsimile No. 01473 660612 |
| Teleprinter No. | |
| 2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning: | |
| <input checked="" type="checkbox"/> the person | <input checked="" type="checkbox"/> the name <input checked="" type="checkbox"/> the address <input type="checkbox"/> the nationality <input type="checkbox"/> the residence |
| Name and Address MURGITROYD & COMPANY Chartered Patent Agents 373 Scotland Street Glasgow G5 8QA United Kingdom | State of Nationality |
| | State of Residence |
| | Telephone No. 141 307 8400 |
| | Facsimile No. 141 307 8401 |
| Teleprinter No. | |
| 3. Further observations, if necessary: | |
| 4. A copy of this notification has been sent to: | |
| <input checked="" type="checkbox"/> the receiving Office | <input type="checkbox"/> the designated Offices concerned |
| <input type="checkbox"/> the International Searching Authority | <input checked="" type="checkbox"/> the elected Offices concerned |
| <input checked="" type="checkbox"/> the International Preliminary Examining Authority | <input type="checkbox"/> other: |

| | |
|---|----------------------------------|
| The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland | Authorized officer S. Buttay |
| Facsimile No.: (41-22) 740.14.35 | Telephone No.: (41-22) 338.83.38 |

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

MURGITROYD & COMPANY
Chartered Patent Agents
373 Scotland Street
Glasgow G5 3QA
GRANDE BRETAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing
(day/month/year) 06.12.2001

Applicant's or agent's file reference

P2662PC/TIPD P2662

IMPORTANT NOTIFICATION

International application No.
PCT/GB00/03223International filing date (day/month/year)
18/08/2000Priority date (day/month/year)
19/08/1999

Applicant

IMMUNOBIOLOGY LIMITED et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/



European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer

Digiusto, M

Tel. +49 89 2399-8152





PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

| | | | |
|---|---|--|--|
| Applicant's or agent's file reference P2662PC/TIPD | | FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) | |
| International application No. PCT/GB00/03223 | International filing date (day/month/year) 18/08/2000 | Priority date (day/month/year) 19/08/1999 | |
| International Patent Classification (IPC) or national classification and IPC A61K39/00 | | | |
| Applicant IMMUNOBIOLOGY LIMITED et al. | | | |
| <p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 8 sheets, including this cover sheet.</p> <p><input checked="" type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of 4 sheets.</p> | | | |
| <p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"> I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input checked="" type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability IV <input type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input checked="" type="checkbox"/> Certain defects in the international application VIII <input checked="" type="checkbox"/> Certain observations on the international application | | | |
| Date of submission of the demand 13/03/2001 | | Date of completion of this report 06.12.2001 | |
| Name and mailing address of the International preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465 | | Authorized officer Barz, W Telephone No. +49 89 2399 /320  | |

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/GB00/03223

1. Basis of the report

1. With regard to the elements of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17):*)

Description, pages:

1-15 as originally filed

Claims, No.:

1-22 as received on 12/11/2001 with letter of 12/11/2001

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
☐ the language of publication of the international application (under Rule 48.3(b)).
☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
☐ filed together with the international application in computer readable form.
☐ furnished subsequently to this Authority in written form.
☐ furnished subsequently to this Authority in computer readable form.
☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/GB00/03223

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 6, 19-21 (IA).

because:

☒ the said international application, or the said claims Nos. 19-21 (IA) relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 6 are so unclear that no meaningful opinion could be formed (*specify*):
see separate sheet

☒ the claims, or said claims Nos. 6 are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

| | |
|-------------------------------|-----------------------------|
| Novelty (N) | Yes: Claims 12 |
| | No: Claims 1-5, 7-11, 13-22 |
| Inventive step (IS) | Yes: Claims |
| | No: Claims 1-5, 7-22 |
| Industrial applicability (IA) | Yes: Claims 1-5, 7-18, 22 |

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. **PCT/GB00/03223**

No: Claims

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

INTERNATIONAL PRELIMINARY International application No. PCT/GB00/03223
EXAMINATION REPORT - SEPARATE SHEET

ITEM I:

The amendments to the claims fulfill the requirements of Article 34 (2)(b) PCT.

ITEM III:

1. **Claim 6** is too unclear to allow examination of said claim, because the expression "cells are dried in the absence of added extra-cellular carbohydrate glassy stabilizing matrix" is neither clear nor supported by the description (Article 6 PCT). Consequently, no opinion will be formulated with respect to said claim (Article 34(4)(a)(ii) PCT).
2. **Claims 19-21** relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT). See, however, item V-3. below.

ITEM V:

Reference is made to the following document:

D1: WO 98 24882 A (QUADRANT HOLDINGS CAMBRIDGE LIMITED),
11 June 1998, cited in the application.

1. **NOVELTY**

Claims 1-5, 7-11, 13-22 do not meet the requirements of Article 33(2) PCT for the following reasons:

- 1.1 A method for producing a vaccine composition comprising all the features of **claim 1** of the present application is disclosed in document D1 (page 4, lines 10-19; page 6, lines 19-21; page 21, lines 4-6). Therefore, the subject-matter of present claim 1 is not novel in the sense of Article 33(2) PCT.

INTERNATIONAL PRELIMINARY

International application No. PCT/GB00/03223

EXAMINATION REPORT - SEPARATE SHEET

The International Preliminary Examining Authority does not agree with the Applicant's argument (provided with the letter dated 12.11.01) that the compositions of D1 would not be directed to vaccines and that the disclosure of D1 would be restricted to disclosing methods of inducing trehalose production in cells. In contrast, in addition to disclosing methods of producing dried, stabilized prokaryotic cells, D1 also discloses the development of "live bacterial vaccines in a dry stable form" (page 6, lines 19-21). Furthermore, it is known from D1 that "the prokaryotic cells are to be used as vaccines, and thus as an immunogenic agent" (page 21, lines 4-5). Consequently, D1 discloses all features of present claim 1, thereby destroying novelty (Article 33(2) PCT) of said claim.

- 1.2 The dependent **claims 2-5, 7-11, and 13** are also not novel (Article 33(2) PCT), because their additional features also disclosed in D1 (page 4, lines 10-19; page 7; lines 25-30; page 10; lines 6-10; example 4; claims 2-3, 7-8, 11).
- 1.3 Similarly, vaccine compositions comprising all features of present **claims 14-18** are also known from D1 (abstract; page 4, lines 10-13; page 6, lines 19-21; page 21, lines 4-6; claims 12 and 21), thereby destroying novelty of said claims (Article 33(2) PCT).
- 1.4 Analogous objections apply to the subject-matter of present **claims 19-21**, because the immunization of animals by administering the vaccine composition is disclosed in D1 (page 6, lines 19-21; page 21, lines 4-6).

Again, the International Preliminary Examining Authority does not agree with the Applicant's argument (see letter of 12.11.01) that "D1 makes no mention of the use of the stabilised dried cells as vaccines". In contrast, such a use is clearly known from D1 (see page 6, lines 19-21, and page 21, lines 4-5).

Concerning the Applicant's argument (letter of 12.11.01) that the dried, stabilised prokaryotic cells which have been stabilised by trehalose induction would be more immunogenic than fresh live cells, such increase in immunogenicity cannot contribute to novelty of the present application, because it is no feature of the present claims.

INTERNATIONAL PRELIMINARY

International application No. PCT/GB00/03223

EXAMINATION REPORT - SEPARATE SHEET

- 1.5 Finally, the genetically modified prokaryotic cell of present **claim 22** is not novel in the sense of Article 33(2) PCT, because it is also known from D1 (page 7, lines 4-6 and 27-30; page 10, lines 8-10).
- 1.6 The remaining **claim 12** is novel, because none of the available prior art documents discloses the same combination of features as said claim.

2. INVENTIVE STEP

However, **claim 12** does not appear to be inventive in the sense of Article 33(3) PCT for the following reason:

Compared to the subject-matter of document D1, which is considered to represent the closest prior art, the method of present **claim 12** differs only in that the induced prokaryotic cells are killed prior to use in the vaccine composition. However, said difference is a obvious routine modification which falls within the scope of the customary practice followed by persons skilled in the art, especially as the advantages thus achieved can readily be foreseen. Consequently, the subject-matter of claim 12 appears to lack an inventive step (Article 33(3) PCT).

3. INDUSTRIAL APPLICABILITY

For the assessment of the present **claims 19-21** on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

INTERNATIONAL PRELIMINARY

International application No. PCT/GB00/03223

EXAMINATION REPORT - SEPARATE SHEET

4. P-DOCUMENTS

Document D1 was published after the priority date, but before the filing date of the present application. Therefore, it is only relevant for those parts of the present application, if any, which do not have a valid claim to priority.

ITEM VII:

The passages on page 2, lines 2 and 22, as well as on page 5, line 32, refer to unpublished documents, in contrast to the requirement that only published documents should be referred to (PCT Guidelines II-4.18).

ITEM VIII:

1. **Claim 7** is not clear in the sense of Article 6 PCT, because protozoa and fungi do not belong to the prokaryotic organisms (see biology textbooks).
2. **Claim 13** is not clear (Article 6 PCT), because it is not apparent what the expression "in vitro" means in the context of prokaryotic cells (killed prokaryotes?).

1 CLAIMS

2

3 1. A method for producing a vaccine composition
4 containing an immunogenic determinant as the
5 active ingredient, characterised in that the
6 method comprises the steps of:

- 7 a. treating procaryotic cells under
8 conditions such that an increase of the
9 concentration of trehalose within
10 procaryotic cells is induced;
11 b. using the induced cells containing
12 trehalose as the immunogenic determinant
13 in the production of a vaccine
14 composition.

15

16 2. A method as claimed in claim 1, characterised
17 in that the treatment of the procaryotic cells
18 is carried out to achieve a concentration of
19 trehalose within the cells of at least 10mM.

20

21 3. A method as claimed in either of claims 1 or 2,
22 characterised in that the increase in
23 concentration of trehalose is achieved by
24 synthesis of trehalose within the cell.

25

26 4. A method as claimed in any one of the preceding
27 claims, characterised in that the condition
28 causing the increase of trehalose concentration
29 within the cells is heat, osmotic shock,
30 suppression of degradation of trehalose, or
31 genetically engineered constitutive synthesis
32 of trehalose within the cells.

AMENDED SHEET

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Emof nr. 2181 P.114

- 1 5. A method as claimed in any one of the preceding
2 claims, characterised in that the induced cells
3 containing the trehalose are dried prior to
4 their use in the production of the vaccine
5 composition.
6
- 7 6. A method as in claim 5, characterised in that
8 the cells are dried in the absence of added
9 extra-cellular carbohydrate glassy stabilising
10 matrix.
11
- 12 7. A method as claimed in any one of the preceding
13 claims, characterised in that the procaryotic
14 cells are bacteria, protozoa or fungi.
15
- 16 8. A method as claimed in any one of the preceding
17 claims, characterised in that the procaryotic
18 cells are treated by cultivating them in a
19 medium containing one or more solutes and
20 having an osmolarity of at least 350 mOsmoles.
21
- 22 9. A method as claimed in claim 8, characterised
23 in that the solute is selected from a sodium,
24 potassium, calcium and / or ammonium salt.
25
- 26 10. A method as claimed in claim 1, characterised
27 in that the procaryotic cell has been modified
28 so as to synthesise trehalose.
29
- 30 11. A method as claimed in claim 1, characterised
31 in that the treatment of the cells is carried

AMENDED SHEET

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- 1 out to achieve a concentration of trehalose
2 within the cells of at least 100mM.
3
4 12. A method as claimed in any one of the preceding
5 claims, characterised in that the procaryotic
6 cells containing the induced trehalose are
7 killed prior to use in the vaccine composition.
8
9 13. A method as claimed in any one of the preceding
10 claims, characterised in that the treatment of
11 the procaryotic cells is carried out in vitro.
12
13 14. A vaccine composition comprising an immunogenic
14 determinant, characterised in that the
15 immunogenic determinant includes a procaryotic
16 cell or cell residue which contains at least
17 10mM of trehalose within the cell.
18
19 15. A vaccine composition characterised in that it
20 contains an immunogenic determinant produced by
21 the method of any of claims 1 to 13.
22
23 16. A vaccine composition as claimed in either of
24 claims 14 or 15, characterised in that it
25 contains an adjuvant for the immunogenic
26 determinant.
27
28 17. A vaccine composition as claimed in any one of
29 claims 14 to 16, characterised in that it
30 contains an aqueous carrier.
31

12-11-2001

12-NOV-01 16:16

FROM MURGITROYD

(MON) 2/11/02 9:25/ST. 9:23/NO. 4860129901 P 15

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T-451 P.07/08

F-401 GB0003223

- 1 18. A vaccine composition as claimed in any one of
2 claims 14 to 17, characterised in that the
3 induced cells containing trehalose are dried in
4 the presence of a non-reducing carbohydrate to
5 provide a storage stable but viable immunogenic
6 determinant for storage prior to use in a
7 vaccine composition.
8
- 9 19. The use of a composition as claimed in any one
10 of claims 14 to 18 immunise an animal.
11
- 12 20. A method for treating an animal with a vaccine,
13 characterised in that a pharmaceutically
14 effective amount of a vaccine composition as
15 claimed in any one of claims 14 to 18 is
16 administered to the animal to elicit an immune
17 response in the animal.
18
- 19 21. A method as claimed in claim 20, characterised
20 in that the vaccine composition is administered
21 by injection.
22
- 23 22. A procaryotic cell which has had its genetic
24 structure modified so as to remove or inhibit
25 that portion of the genetic structure which
26 inhibits or restricts the synthesis of
27 trehalose by the cell whereby the cell
28 constitutively synthesises trehalose within the
29 cell as it grows.

AMENDED SHEET

From 202-842-8465 12/11/2001 17:02

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PCT

REC'D 11 DEC 2001

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

| | | |
|--|---|---|
| Applicant's or agent's file reference P2662PC/TIPD | FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) | |
| International application No. PCT/GB00/03223 | International filing date (day/month/year) 18/08/2000 | Priority date (day/month/year) 19/08/1999 |
| International Patent Classification (IPC) or national classification and IPC A61K39/00 | | |
| Applicant IMMUNOBIOLOGY LIMITED et al. | | |



- This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
- This REPORT consists of a total of 8 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

 These annexes consist of a total of 4 sheets.

- This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☒ Certain defects in the international application
- VIII ☒ Certain observations on the international application

| | |
|---|--|
| Date of submission of the demand 13/03/2001 | Date of completion of this report 06.12.2001 |
| Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465 | Authorized officer Barz, W Telephone No. +49 89 2399 7320  |

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/03223

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

Description, pages:

1-15 as originally filed

Claims, No.:

1-22 as received on 12/11/2001 with letter of 12/11/2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/03223

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 6, 19-21 (IA).

because:

☒ the said international application, or the said claims Nos. 19-21 (IA) relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 6 are so unclear that no meaningful opinion could be formed (*specify*):
see separate sheet

☒ the claims, or said claims Nos. 6 are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

| | | | |
|-------------|------|--------|------------------|
| Novelty (N) | Yes: | Claims | 12 |
| | No: | Claims | 1-5, 7-11, 13-22 |

| | | | |
|---------------------|------|--------|-----------|
| Inventive step (IS) | Yes: | Claims | |
| | No: | Claims | 1-5, 7-22 |

| | | | |
|-------------------------------|------|--------|---------------|
| Industrial applicability (IA) | Yes: | Claims | 1-5, 7-18, 22 |
|-------------------------------|------|--------|---------------|

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/GB00/03223

No: Claims

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/GB00/03223

ITEM I:

The amendments to the claims fulfill the requirements of Article 34 (2)(b) PCT.

ITEM III:

1. **Claim 6** is too unclear to allow examination of said claim, because the expression "cells are dried in the absence of added extra-cellular carbohydrate glassy stabilizing matrix" is neither clear nor supported by the description (Article 6 PCT). Consequently, no opinion will be formulated with respect to said claim (Article 34(4)(a)(ii) PCT).
2. **Claims 19-21** relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT). See, however, item V-3. below.

ITEM V:

Reference is made to the following document:

D1: WO 98 24882 A (QUADRANT HOLDINGS CAMBRIDGE LIMITED),
11 June 1998, cited in the application.

1. NOVELTY

Claims 1-5, 7-11, 13-22 do not meet the requirements of Article 33(2) PCT for the following reasons:

- 1.1 A method for producing a vaccine composition comprising all the features of **claim 1** of the present application is disclosed in document D1 (page 4, lines 10-19; page 6, lines 19-21; page 21, lines 4-6). Therefore, the subject-matter of present claim 1 is not novel in the sense of Article 33(2) PCT.

The International Preliminary Examining Authority does not agree with the Applicant's argument (provided with the letter dated 12.11.01) that the compositions of D1 would not be directed to vaccines and that the disclosure of D1 would be restricted to disclosing methods of inducing trehalose production in cells. In contrast, in addition to disclosing methods of producing dried, stabilized prokaryotic cells, D1 also discloses the development of "live bacterial vaccines in a dry stable form" (page 6, lines 19-21). Furthermore, it is known from D1 that "the prokaryotic cells are to be used as vaccines, and thus as an immunogenic agent" (page 21, lines 4-5). Consequently, D1 discloses all features of present claim 1, thereby destroying novelty (Article 33(2) PCT) of said claim.

- 1.2 The dependent **claims 2-5, 7-11, and 13** are also not novel (Article 33(2) PCT), because their additional features also disclosed in D1 (page 4, lines 10-19; page 7; lines 25-30; page 10; lines 6-10; example 4; claims 2-3, 7-8, 11).
- 1.3 Similarly, vaccine compositions comprising all features of present **claims 14-18** are also known from D1 (abstract; page 4, lines 10-13; page 6, lines 19-21; page 21, lines 4-6; claims 12 and 21), thereby destroying novelty of said claims (Article 33(2) PCT).
- 1.4 Analogous objections apply to the subject-matter of present **claims 19-21**, because the immunization of animals by administering the vaccine composition is disclosed in D1 (page 6, lines 19-21; page 21, lines 4-6).

Again, the International Preliminary Examining Authority does not agree with the Applicant's argument (see letter of 12.11.01) that "D1 makes no mention of the use of the stabilised dried cells as vaccines". In contrast, such a use is clearly known from D1 (see page 6, lines 19-21, and page 21, lines 4-5).

Concerning the Applicant's argument (letter of 12.11.01) that the dried, stabilised prokaryotic cells which have been stabilised by trehalose induction would be more immunogenic than fresh live cells, such increase in immunogenicity cannot contribute to novelty of the present application, because it is no feature of the present claims.

- 1.5 Finally, the genetically modified prokaryotic cell of present **claim 22** is not novel in the sense of Article 33(2) PCT, because it is also known from D1 (page 7, lines 4-6 and 27-30; page 10, lines 8-10).
- 1.6 The remaining **claim 12** is novel, because none of the available prior art documents discloses the same combination of features as said claim.

2. INVENTIVE STEP

However, **claim 12** does not appear to be inventive in the sense of Article 33(3) PCT for the following reason:

Compared to the subject-matter of document D1, which is considered to represent the closest prior art, the method of present **claim 12** differs only in that the induced prokaryotic cells are killed prior to use in the vaccine composition. However, said difference is a obvious routine modification which falls within the scope of the customary practice followed by persons skilled in the art, especially as the advantages thus achieved can readily be foreseen. Consequently, the subject-matter of claim 12 appears to lack an inventive step (Article 33(3) PCT).

3. INDUSTRIAL APPLICABILITY

For the assessment of the present **claims 19-21** on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/GB00/03223

4. P-DOCUMENTS

Document D1 was published after the priority date, but before the filing date of the present application. Therefore, it is only relevant for those parts of the present application, if any, which do not have a valid claim to priority.

ITEM VII:

The passages on page 2, lines 2 and 22, as well as on page 5, line 32, refer to unpublished documents, in contrast to the requirement that only published documents should be referred to (PCT Guidelines II-4.18).

ITEM VIII:

1. **Claim 7** is not clear in the sense of Article 6 PCT, because protozoa and fungi do not belong to the prokaryotic organisms (see biology textbooks).
2. **Claim 13** is not clear (Article 6 PCT), because it is not apparent what the expression "in vitro" means in the context of prokaryotic cells (killed prokaryotes?).

CLAIMS:

1. A method for producing a vaccine composition
containing an immunogenic determinant as the active
ingredient, characterised in that the method
comprises the steps of:
 - a. treating procaryotic cells under conditions such
that an increase of the concentration of trehalose
within procaryotic cells is induced;
 - b. using the induced cells containing trehalose as the
immunogenic determinant in the production of a
vaccine composition.
2. A method as claimed in claim 1, characterised in that
the treatment of the procaryotic cells is carried out
to achieve a concentration of trehalose within the
cells of at least 10mM.
3. A method as claimed in either of claims 1 or 2,
characterised in that the increase in concentration
of trehalose is achieved by synthesis of trehalose
within the cell.
4. A method as claimed in any one of the preceding
claims, characterised in that the condition causing
the increase of trehalose concentration within the
cells is heat, osmotic shock, suppression of
degradation of trehalose, or genetically engineered
constitutive synthesis of trehalose within the cells.
5. A method as claimed in any one of the preceding
claims, characterised in that the induced cells

containing the trehalose are dried prior to their use in the production of the vaccine composition.

- 5 6. A method as in claim 5, characterised in that the cells are dried in the absence of added extra-cellular carbohydrate glassy stabilising matrix.
- 10 7. A method as claimed in any one of the preceding claims, characterised in that the procaryotic cells are bacteria, protozoa or fungi.
- 15 8. A method as claimed in any one of the preceding claims, characterised in that the procaryotic cells are treated by cultivating them in a medium containing one or more solutes and having an osmolarity of at least 350 mOsmoles.
- 20 9. A method as claimed in claim 8, characterised in that the solute is selected from a sodium, potassium, calcium and/or ammonium salt.
- 25 10. A method as claimed in claim 1, characterised in that the procaryotic cell has been modified so as to synthesise trehalose.
- 30 11. A method as claimed in claim 1, characterised in that the treatment of the cells is carried out to achieve a concentration of trehalose within the cells of at least 100mM.
12. A method as claimed in any one of the preceding claims, characterised in that the procaryotic cells

containing the induced trehalose are killed prior to use in the vaccine composition.

- 5 13. A method as claimed in any one of the preceding claims, characterised in that the treatment of the procaryotic cells is carried out in vitro.
- 10 14. A method as claimed in claim 1, substantially as hereinbefore described in any one of the examples.
- 15 15. A vaccine composition comprising an immunogenic determinant, characterised in that the immunogenic determinant includes a procaryotic cell or cell residue which contains at least 10mM of trehalose within the cell.
- 20 16. A vaccine composition characterised in that it contains an immunogenic determinant produced by the method of any of claims 1 to 14.
- 25 17. A vaccine composition as claimed in either of claims 15 or 16, characterised in that it contains an adjuvant for the immunogenic determinant.
- 30 18. A vaccine composition as claimed in any one of claims 15 to 17, characterised in that it contains an aqueous carrier.
19. The use of a composition as claimed in claim 15 to immunise an animal.

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WO 01/13942 A2

(54) Title: **TREHALOSE PRODUCING CELLS AS VACCINES**

(57) Abstract: The present invention relates to methods for using procaryotic cells which have been modified or induced to synthesise trehalose as vaccines and to vaccine compositions obtained thereby.

TITLE: TREHALOSE PRODUCING CELLS AS VACCINES

This invention relates to the field of vaccines. More specifically, it relates to methods of producing vaccines
5 of trehalose containing procaryotic cells and the compositions obtained thereby.

BACKGROUND TO THE INVENTION:

10 Procaryotic cells, particularly bacteria, are widely and increasingly used in medical, agricultural and industrial applications. Agricultural, or environmental, applications include biopesticides and bioremediation. Medical applications include use of bacteria in vaccines
15 as well as for production of pharmaceutical products for other treatments.

For the procaryotic cells to be used effectively, both in terms of desired results and cost, the cells must be able
20 to be stored for significant periods of time whilst preserving their viability. The term viability is used herein to denote that the cells manifest the features of a functioning living organism, such as metabolism and cell division.

25 Methods for preserving live procaryotic cells suffer from several serious drawbacks, such as being energy-intensive and requiring cold storage. Thus, freeze-drying is often used for preservation and storage of procaryotic cells.
30 However, it has the undesirable characteristic of significantly reducing viability of the cells, as well as being time- and energy-intensive and thus expensive.

PCT GB97/03375 describes a process of stabilising procaryotic cells by the induction of trehalose synthesis and the drying of the resulting cells in a glassy carbohydrate matrix. This process gives stabilised cells that can be stored at ambient temperatures without loss of viability. Trehalose, (α -D-glucopyranosyl- α -D-glucopyranoside), is a naturally occurring, non-reducing disaccharide which was initially found to be associated with the prevention of desiccation damage in certain plants and animals which can dry out without damage and can revive when re-hydrated. Trehalose has been shown to be useful in preventing denaturation of proteins, viruses and foodstuffs during desiccation, see U.S. Patents Nos. 4,891,319; 5,149,653; 5,026,566; Colaco et al. (1992) *Bio/Tech.* 10:1007-1011.

Trehalose synthesis in procaryotic cells is induced by a number of methods including osmotic shock which induces the endogenous production of trehalose, Welsh et al. (1991) *J. Gen. Microbiol.* 137:745-750.

PCT application No. GB94/01556 describes a process of improving the viability of bacterial dried cells by the induction of trehalose synthesis by nutrient limitation, heat shock or osmoadaptation. PCT application No. GB97/03375 describes a method for the preservation of procaryotic cells by the drying of cells in a carbohydrate matrix after the induction of trehalose synthesis. The latter invention provides compositions of dried cells that can be stored at ambient temperatures and thus enable a number of industrial applications.

Surprisingly, we have now found that the dried, stabilised procaryotic cells produced by the above methods, are more immunogenic than fresh live cells and hence have particular value as the immunogenic determinant active component in vaccine compositions. Furthermore, we have also found that this increased immunogenicity of the stabilised procaryotic cells is not dependent on the drying process considered essential in the above stabilisation processes, but results from the induction of trehalose synthesis. Although more pronounced with dried cells, this increased immunogenicity is also seen in cells induced to produce trehalose but which have not been subjected to a drying process.

15

SUMMARY OF THE INVENTION:

The present invention thus provides a method for producing a vaccine composition, which comprises the steps of:

- 20 a. Treating procaryotic cells in vitro under conditions such that an increase of the concentration of trehalose within procaryotic cells is induced, preferably by the synthesis of trehalose within the cell;
- 25 b. using the induced cells containing trehalose as the immunogenic determinant in the production of a vaccine composition.

Preferably, the treatment of the procaryotic cells is carried out to achieve a concentration of trehalose within the cells of at least 10mM.

30

Preferably, the induced cells containing the trehalose are dried prior to their use in the production of the vaccine composition, notably in the presence of a non-reducing carbohydrate such as trehalose to provide a storage stable
5 but viable immunogenic determinant for storage prior to use in a vaccine composition.

The invention also provides a vaccine composition containing an immunogenic determinant, characterised in
10 that the immunogenic determinant has been made by the method of the invention.

The invention also provides a method for immunising an animal which comprises administering a pharmaceutically
15 effective amount of a vaccine composition of the invention to an animal sufficient to elicit an immune response in the animal.

Preferably, the vaccine composition contains an adjuvant
20 for the immunogenic determinant, is put up in an aqueous carrier medium and is administered by injection.

The procaryotic cells for use in the present invention are ones which are capable of synthesising trehalose. This
25 ability can be native or can be conferred by recombinant techniques. The ability of a procaryotic cell to synthesise trehalose can be determined by measuring trehalose concentration as described below.

30 The term procaryotic is used herein to denote cells that exhibit characteristics of procaryotes, which are typically unicellular organisms, lack organelles (such as

mitochondria, chloroplasts, and Golgi apparatus), lack a cytoskeleton and lack a discrete nucleus. Examples of procaryotic cells for present use include bacteria, such as eubacteria, cyanobacteria and prochlorophytes; 5 archaeobacteria; and other microorganisms such as rickettsias, mycoplasmas, spiroplasmas, and chlamydiae. Preferred procaryotic cells for present use are bacteria.

In general, any procaryotic cell or mixture of cells, 10 particularly bacteria, containing trehalose synthase genes should be capable of synthesising trehalose. Bacteria have two genes involved in trehalose synthesis (i.e. T-Phosphate synthase and T-P phosphatase), whereas yeasts have at least three genes and combinations of these genes 15 may be used to enable trehalose synthesis. Examples of bacteria that contain the trehalose synthase gene include, but are not limited to, Enterobacteriaceae, such as *Salmonella* and *Escherichia* (e.g., *S. typhimurium* and *E.coli*); halophilic and halotolerant bacteria, such as 20 *Ectothriorhodospira* (e.g., *E.halochloris*); micrococcaceae, such as *Micrococcus* (e.g., *M.luteus*); *Rhizobium* species such as *R. japonicum* and *R. leguminosarum* bv *phaseoli*; *Cyanobacteria*; *Mycobacteria* species such as *M. tuberculosis*, *M. bovis*, and *M. smegmatis*.

25 Procaryotic cells can be induced to synthesise trehalose by culturing the cells in vitro under stressful conditions, e.g., osmotic shock, heat or oxygen limitation (shock), carbon/nitrogen starvation, or any combination of 30 the above. Suitable conditions include those heat shock and other conditions described, for example, in PCT applications Nos. GB94/01556 and GB97/03375.

Alternatively, use of inhibitors, such as validomycin, of enzyme(s) such as trahalase involved in trehalose degradation may also result in an increase of trehalose concentration within the cells. Alternatively, the genetic structure of the procaryotic organism may be modified to remove or inhibit that portion of the genetic structure which inhibits or restricts the synthesis of trehalose within the cell so that the cells constitutively synthesise trehalose as they are cultured without the need to apply external stimuli. Such genetic modification can be achieved using any suitable technique. For convenience, the invention will be described hereinafter in terms of the use of external stimuli to induce the production of trehalose within the cell, rather than the use of a procaryotic cell which has had its genetic structure modified.

The term osmotic shock is used herein to denote that the solute concentration in the growth medium within which the cells are cultivated is above the level at which a cell exists and/or grows in its native environment. The solute may be a mixture of salts and the concentration is typically from 0.2 to 0.5 Mols above the level at which the cell is normally cultivated.

We believe that induction of trehalose sythesis under stressful conditions may also induce synthesis or accumulation of other molecules that may be beneficial for preservation, such as betaine and chaperonins or which enhance the vaccine action of the induced cells.

For bacteria, particularly *Escherichia*, trehalose

synthesis is preferably induced by growing the cell(s) in conditions of high osmolarity, i.e., salt concentrations sufficient to stimulate trehalose production. To induce trehalose synthesis by osmotic shock, the total
5 concentration of salt(s) in the medium should be at least about 0.2M, preferably at least about 0.4M, more preferably at least about 0.5M. The total concentration of salt(s) should not exceed 0.6M, since above this level trehalose synthesis declines in *E.coli*. The salt
10 concentrations correspond to osmolarities of at least about 350 mOsmoles to about 1.5 Osmoles, preferably at least about 400 mOsmoles to 1 Osmole, most preferably 250 mOsmoles to 500 mOsmoles. Generally, a minimum osmolarity of about 200 mOsmoles is required as this will usually
15 provide a higher concentration of solute than that under which the cells are usually cultivated.

The necessary solute can be provided by the use of a single salt, for example, 200mM NaCl KCl and/or CaCl₂.
20 (NH₄)₂SO₄ may also be used, however only about one half of the amount of trehalose is produced compared to that produced in the presence of KCl, NaCl and/or CaCl₂. A mixture of salts can also be used. In addition, when used to increase the osmolarity of the medium, a non-penetrant
25 solute such as sorbitol and/or glucose can contribute to the stimulation of trehalose synthesis.

The salt concentration (i.e., osmolarity) required to stimulate and/or induce trehalose sythesis will depend
30 upon the genus, species, and/or strain of the procaryotic cell used. Preferably, cell(s) are grown in a minimal medium containing solutes and commercially available

minimal media can be supplemented with desired salts and/or other solutes. The use of a minimal medium is not essential and defined media can also be used. The time required to initiate and achieve the desired level of trehalose concentration within the cells will vary depending on the level of osmolarity as well as the genus, species and/or strain of procaryotic cell used. Trehalose synthesis will generally begin within an hour of placing cells in conditions designed to stimulate trehalose production. Generally, in *E.coli* the synthesis of trehalose reaches a maximum at about 15-20 hours.

Synthesis of trehalose may also be stimulated using recombinant methods which are well known in the art. For instance, procaryotic cells can be transfected with a DNA plasmid comprising a DNA sequence encoding the appropriate trehalose synthase gene. The gene in turn is operatively linked to a suitable promoter, which can be constitutive or inducible. Suitable recombinant techniques are described in, for example, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook et al., 1989).

The concentration of trehalose synthesised within the procaryotic cells can be measured using any suitable assay technique, for example by high pressure liquid chromatography (HPLC), coupled with electro-chemical detection and glucose assay (Trinder assay using trehalase) for quantitative enzymatic determination of trehalose.

Thin layer chromatography can be used as a qualitative method for the separation of different carbohydrates.

Refractive index detection provides another means of detecting sugars quantitatively.

In measuring trehalose by HPLC, cells are disrupted and trehalose preferentially solubilized in 70% ethanol, followed by removing triglycerides by chloroform extraction. Trehalose concentration is determined by multiplying trehalose concentration (as determined by a standard curve) by the fraction of final volume of supernatant divided by pellet volume. A more detailed description of this assay is provided in Example 1.

Preferably, the synthesis is carried out to provide a concentration of trehalose within the cells of at least about 10mM, for example at least about 30mM, preferably at least about 50mM, notably at least about 100mM.

Thus, in a preferred aspect the invention includes culturing the procaryotic cells under conditions that stimulate intracellular production of trehalose, wherein intracellular concentration of trehalose reaches at least about 10mM, preferably at least about 30mM, more preferably at least about 50mM, notably at least about 100mM. It is particularly preferred that the concentration be at least about 150mM.

The time required for stimulating trehalose synthesis depends, inter alia, on the nature of the procaryotic cells (including genus, species, and/or strain) and the conditions under which trehalose induction occurs (i.e., whether by osmotic shock, oxygen deprivation, etc.). For trehalose induction by osmotic shock, the time required

for maximum concentration of trehalose in turn depends on the degree of osmolarity as well as the particular salts used. The optimum conditions for trehalose synthesis can readily be determined by simple trial and errors tests.

5

The cultivated procaryotic cells containing the intracellular trehalose may then be frozen for storage before use as a vaccine. Alternatively storage of the vaccine can be effected by culturing the procaryotic cells under conditions that increase trehalose concentration to a level effective to increase storage stability, mixing the cells with a drying solution which contains a stabilising agent, and drying the cells under conditions such that a glass is produced having less than about 5% residual moisture. If a killed vaccine rather than a live vaccine is required, the cells may be killed by any suitable method, for example chemical fixation and radiation prior to processing for storage. Though the procaryotic cells may be used as the sole immunogenic determinant active ingredient in the vaccine, an adjuvant may be added in an amount sufficient to enhance the immune response to the procaryotic vaccine. The adjuvant can be added to the procaryotic cells before drying, for example, cholera B toxin sub-unit can be dried simultaneously with *V. cholera*. Alternatively the adjuvant may be obtained and dried separately, and reconstituted along with the procaryotic cells.

Suitable adjuvants include, but are not limited to, aluminium hydroxide, alum, QS-21 (U.S. Pat. No 5,057,540), DHEA (U.S. Pats. Nos. 5,407,684 and 5,077,284) and its derivatives (including salts) and precursors (e.g., DHEA-

S), beta-2 microglobulin (WO 91/16924), muramyl dipeptides, muramyl tripeptides (U.S. Pat. No. 5,171,568), monophosphoryl lipid A (U.S. Pat. No. 4,436,728; WO 92/16231) and its derivatives (e.g., Detox™), and BCG (U.S. Pat. No. 4,726,947). Other suitable adjuvants include aluminium salts, squalene mixtures (SAF-1), muramyl peptide, saponin derivatives, mycobacterium wall preparations, mycolic acid derivatives, non-ionic block copolymer surfactants, Quil A, cholera toxin B sub-unit, polyphosphazene and derivatives, and immunostimulating complexes (ISCOMs) such as those described by Takahashi et al. (1990) *Nature* 344:873-875. The choice of an adjuvant will depend in part on the stability of the vaccine in the presence of the adjuvant, the route of administration, and the regulatory acceptability of the adjuvant, particularly when intended for human use. For instance, alum is approved by the United States Food and Drug Administration (FDA) for use as an adjuvant in humans.

The invention also provides a method for treating an animal with a vaccine of the invention by administering a pharmaceutically acceptable quantity of the vaccine of the invention, optionally in combination with an adjuvant, sufficient to elicit an immune response in the animal. The animal is typically a human. However, the invention can also be applied to the treatment of other mammals such as horses, cattle, goats, sheep or swine, and to the treatment of birds, notably poultry such as chicken or turkeys.

The vaccine compositions of the present invention may be administered by any suitable means, such as orally, by

inhalation, transdermally or by injection and in any suitable carrier medium. However, it is preferred to administer the vaccine as an aqueous composition by injection using any suitable needle or needle-less
5 technique.

The vaccines of the invention may contain any suitable concentration of the induced procaryotic cells. We prefer that the cells are administered at doses in the range of
10 10-600 µg, preferably 10-100 µg, most preferably 25 µg, per Kg of body weight of the animal being treated. It will be appreciated that the vaccine of the invention may be applied as an initial treatment followed by one or more subsequent treatments at the same or a different dosage
15 rate at an interval of from 1 to 26 weeks between each treatment to provide prolonged immunisation against the pathogen.

20

The following examples are provided to illustrate but not limit the invention.

25

Example 1: Induction of trehalose in *E.coli* by osmotic shock:

30

E.coli (NCIMB strain 9484) was cultured in Evans medium (pH 7.0) containing 5mM ammonium chloride. After overnight incubation at 37°C in the initial Evans medium, a 4ml culture of *E.coli* grown in Evans medium under nitrogen limitation was used to inoculate a 200ml culture of Evans medium modified to induce osmotic shock by

increasing the salt concentration (KCl) to 0.5M.

Trehalose concentration was measured by high pressure liquid chromatography (HPLC) analysis and significant increases in trehalose concentrations were observed at 15-17 hours after initiation of osmotic shock, with values peaking at less than 20 hours.

Example 2: Induction of trehalose synthesis in *Salmonella*:

10

Salmonella typhimurium (1344) was grown overnight at 37°C in M9 (minimal) medium with and without 0.5M NaCl. Cells were harvested by centrifugation and analysed for trehalose concentration by HPLC analysis as described in Example 1. Growth in high salt medium showed at 4 to 5 fold induction of trehalose synthesis as compared to the low salt medium.

20

Example 3: Drying of procaryotic cells after induction of trehalose synthesis:

E.coli and *Salmonella typhimurium* were grown overnight at 37°C in M9 (minimal) medium with and without 0.5M NaCl and trehalose synthesis induced as described in examples 1 and 2. The induced bacteria were harvested by centrifugation at 10,000 rpm for 10 minutes and the cell pellets re-suspended in drying solution containing 45% trehalose, 0.1% cmc (sodium carboxymethyl cellulose, Blanose 7HF, Aqualon) to a typical cell density of $0.5-1.2 \times 10^9$ CFU/ml. 300µl and 500µl aliquots were dispensed into 3ml

pharmaceutical vials and dried under vacuum without freezing, overnight at ambient temperature and a vacuum pressure of 30mTorr. Alternatively, the aliquots can be freeze-dried using the following protocol: ramp at
5 2.5°C/min to an initial shelf temperature of -40°C; primary drying was performed at a vacuum pressure of 30mT at -40°C and held for 40 hours; for secondary drying ramp at 0.05°C/min from -40 to 30°C and hold for 12 hours.

10 Example 4: Use of induced procaryotic cells as vaccines:

E.coli and *Salmonella typhimurium* cells were induced to synthesise trehalose as in Examples 1 and 2 and were used to immunise mice and rabbits. Titration of the bacteria
15 showed that a 100 to 1000 fold lower titre of bacteria induced for trehalose synthesis was required to produce an equivalent antibody response in the animals compared to the use of non-induced bacteria. Dried preparations were generally 2-50 fold more effective on a cell number basis
20 at eliciting protective immunity in the immunised animals than non-dried preparations.

Example 5: Use of induced procaryotic cells as vaccines; heat-induced trehalose synthesis:

25

E.coli and *Salmonella typhimurium* (strains as in examples 1 and 2) were grown overnight at 37°C in LB medium. 4ml aliquots of the stationary cultures were used to inoculate 200ml of LB medium in a 2 litre conical flask and the
30 cultures grown for 3hrs at 30°C. The log phase cultures were then raised to 40°C and grown for a further 3hrs before the bacteria were harvested by centrifugation at

- 15 -

10,000 rpm for 10 minutes. A similar protocol was used for the growth and induction of *Mycobacterium Bovis* and *Vaccae* (NCTC 11659) which were grown for 2 days in Sauton's medium before dilution to obtain log phase
5 cultures for heat-induction. Cell pellets were re-suspended in lysis solution containing 0.5% Tween and the trehalose concentration was measured by high pressure liquid chromatography (HPLC) analysis. Typically 3-5 fold increases in trehalose concentrations were observed as
10 compared to cells grown at 30°C alone.

Bacterial cells induced to synthesise trehalose as described above were killed by repeated freeze-thaw cycles and used to immunise rabbits. Antibody titres in the
15 immunised animals were assayed by 10-fold serial dilutions using a dot-blot assay on total cell lysates prepared as described for trehalose analysis above. Animals vaccinated with induced bacteria showed a 10 to 100 fold higher antibody titre than those immunised with non-
20 induced bacteria.

CLAIMS:

1. A method for producing a vaccine composition containing an immunogenic determinant as the active ingredient, characterised in that the method comprises the steps of:
 - a. treating procaryotic cells under conditions such that an increase of the concentration of trehalose within procaryotic cells is induced;
 - b. using the induced cells containing trehalose as the immunogenic determinant in the production of a vaccine composition.
2. A method as claimed in claim 1, characterised in that the treatment of the procaryotic cells is carried out to achieve a concentration of trehalose within the cells of at least 10mM.
3. A method as claimed in either of claims 1 or 2, characterised in that the increase in concentration of trehalose is achieved by synthesis of trehalose within the cell.
4. A method as claimed in any one of the preceding claims, characterised in that the condition causing the increase of trehalose concentration within the cells is heat, osmotic shock, suppression of degradation of trehalose, or genetically engineered constitutive synthesis of trehalose within the cells.
5. A method as claimed in any one of the preceding claims, characterised in that the induced cells

containing the trehalose are dried prior to their use in the production of the vaccine composition.

- 5 6. A method as in claim 5, characterised in that the cells are dried in the absence of added extra-cellular carbohydrate glassy stabilising matrix.
- 10 7. A method as claimed in any one of the preceding claims, characterised in that the procaryotic cells are bacteria, protozoa or fungi.
- 15 8. A method as claimed in any one of the preceding claims, characterised in that the procaryotic cells are treated by cultivating them in a medium containing one or more solutes and having an osmolarity of at least 350 mOsmoles.
- 20 9. A method as claimed in claim 8, characterised in that the solute is selected from a sodium, potassium, calcium and/or ammonium salt.
- 25 10. A method as claimed in claim 1, characterised in that the procaryotic cell has been modified so as to synthesise trehalose.
- 30 11. A method as claimed in claim 1, characterised in that the treatment of the cells is carried out to achieve a concentration of trehalose within the cells of at least 100mM.
12. A method as claimed in any one of the preceding claims, characterised in that the procaryotic cells

containing the induced trehalose are killed prior to use in the vaccine composition.

5 13. A method as claimed in any one of the preceding claims, characterised in that the treatment of the procaryotic cells is carried out in vitro.

14. A method as claimed in claim 1, substantially as hereinbefore described in any one of the examples.

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15. A vaccine composition comprising an immunogenic determinant, characterised in that the immunogenic determinant includes a procaryotic cell or cell residue which contains at least 10mM of trehalose within the cell.

15

16. A vaccine composition characterised in that it contains an immunogenic determinant produced by the method of any of claims 1 to 14.

20

17. A vaccine composition as claimed in either of claims 15 or 16, characterised in that it contains an adjuvant for the immunogenic determinant.

25 18. A vaccine composition as claimed in any one of claims 15 to 17, characterised in that it contains an aqueous carrier.

30 19. The use of a composition as claimed in claim 15 to immunise an animal.

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(54) Title: TREHALOSE PRODUCING PROKARYOTIC CELLS AS VACCINES

(57) Abstract: The present invention relates to methods for using procaryotic cells which have been modified or induced to synthe-
sise trehalose as vaccines and to vaccine compositions obtained thereby.

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A. CLASSIFICATION OF SUBJECT MATTER

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, CANCERLIT, LIFESCIENCES, EMBASE, CHEM ABS Data, SCISEARCH, WPI Data, EPO-Internal, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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